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DEVELOPMENT OF VACCINES TO PREVENT WOUND INFECTIONS DUE TO ANAE--ETC(U)
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

To determine the role of Bacteroides fragilis in the development of infectious complications following hysterectomy, we have measured serum antibody to the capsular polysaccharide with a radioactive antigen-binding assay in 53 women before and several days after surgery. Patients with post-operative abscess formation had significantly greater mean increase in antibody concentration (4.91 µg/ml) than those without complications (0.62 µg/ml), with wound infection (1.90 µg/ml), with pelvic cellulitis

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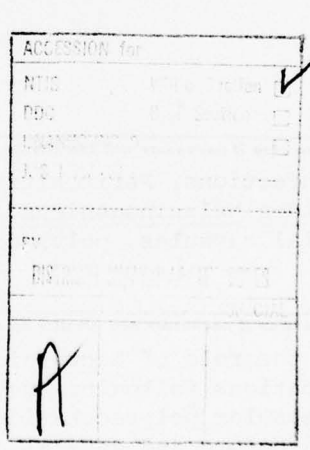
(0.63 µg/ml), and with febrile morbidity (0.95 µg/ml). There were no significant difference among the latter four groups. We conclude that B. fragilis, may play an important role in abscess formation following hysterectomy, but is infrequently involved in other infectious complications of this procedure.

A modification of the previously described model is reported in which infective material is implanted subcutaneuously into Wistar rats. Studies with this model suggest that host response to bacterial challenge is dependent on the site of infections, and that organotropism of the implanted bacterial species is an important determinant of infection.

An indirect fluorscent antibody (IFA) test was developed using hyper-immune rabbit serum to a purified capsular polysaccharide of Bacteroides melaninogenicus subspecies asaccharolyticus. Twenty-three of twenty-three strains of B. melaninogenicus ss. asaccharolyticus were fluorescence-postiive using this test. All eleven strains of B. melaninogenicus ss. intermedius tested and three strains of B. melaninogenicus ss melaninogenicus were fluorescence-negative. The IFA test demonstrated the presence of a subspecies specific capsular antigen from B. melaninogenicus ss. asaccharolyticus. The capsule was further demonstrated by electron microscopy using ruthenium red, a polysaccharide-staining material.

The enzyme-linked immunoabsorbent assay has been developed to measure specific IgG directed againt a subspecies specific capsular antigen of B. melaninogenicus subspecies asaccharolyticus. With this test we have shown that hyperimmune rabbit serum to strains of the subspecies all contain significant measurable IgG directed against a capsular antigen from one strain. Almost no specific IgG was detected in antiserum made against strains of the other two subspecies (intermedius and melaninogenicus) or in antisera made against several other bacterial species.



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# 5th Annual Report

# Development of Vaccines to Prevent Wound Infections due to Anaerobic Bacteria

## ANNUAL REPORT

(for the period 1 January 1978 to 28 August 1978)

Dennis L. Kasper, M.D.

August 1978

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701.

Contract No. DAMD-17-78C-8019
The Peter Bent Brigham Hospital
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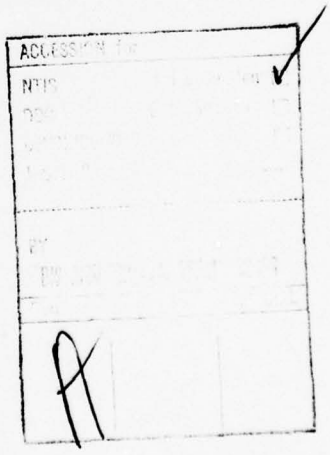

# I. Bacteroides Fragilis

## A. Studies of the antibody response to B. fragilis in infected people

The importance of Bacteroides fragilis in post-operative infections is documented (1-3). Furthermore, the special propensity for this organism to form abscesses has been demonstrated in a rat model (4) and suggested by clinical observations (3,5). In prospective fashion, we collected serologic data to determine the role of B. fragilis in abscess formation following elective surgery (in this group hysterectomy) as well as its role in other infectious or presumably infectious complications of this operative procedure. We have previously documented the association of change in antibody level to the capsular polysaccharide of B. fragilis with isolation of the organism from infected sites.

### Patients and Methods

In the course of a randomized clinical trial of the efficacy of antimicrobial prophylaxis in preventing infections following abdominal or vaginal hysterectomy, serum specimens were collected from potential participants prior to surgery. At time of discharge, a second serum specimen was collected from categories of patients: 1) uncomplicated post-operative course; 2) febrile morbidity (oral temperature $>100^{4}$ F on at least two days following surgery, excluding the first 24 hours); 3) wound infection; 4) pelvic cellulitis; and 5) abscess (cuff abscess of pelvic abscess). Sera were stored at $-20^{\circ}$C until antibody measurements were performed.

During the 22 month period of study, 1217 hysterectomies were performed at the Boston Hospital for Women. In 38 (3.1%) of these, a post-operative cuff abscess or pelvic abscess was documented. For nine of these, paired sera were available for study. Along with these, antibody concentrations were measured in paired specimens, from 44 patients in the other four categories. These specimens were chosen on the basis of availability and without knowledge of any bacteriologic results. Furthermore, antibody levels were determined by a technician who was unaware of the clinical diagnoses of the patients.

Antibodies to the capsular polysaccharide of B. fragilis were quantified by a sensitive radioactive antigen-binding assay (RABA) described by Kasper (6,7).

Differences between pre-surgery and discharge serum antibody concentrations among the five patient groups were evaluated using a one-way analysis of variance (8). Group mean differences were analyzed further using the method of Scheffe (9).

### Results

Table 1 demonstrates the comparability of the five groups at baseline. These baseline antibody concentrations varied widely (2.0 to 17.7 µg/ml), as did final concentrations. (See Table 1).

Table 2 lists the changes in antibody concentrations for each patient, categorized into five groups by diagnosis. (See Table 2). Analysis of variance demonstrated that the five groups differed significantly in their mean changes in antibody concentration between the pre- and post-surgery specimens ($F_{4,48} = 6.54$; $p<0.005$). Further analysis revealed that the mean antibody concentration rise in the abscess group (4.91 µg/ml) was significantly greater than those among patients with no complications (0.62 µg/ml), wound infection (1.90 µg/ml), pelvic cellulitis (0.63 µg/ml), and febrile morbidity (0.95 µg/ml) ($t=4.52$; $p<0.05$). The mean changes in antibody concentrations among the latter four groups of patients were not significantly different from each other ($t= 1.35$; $0.20<p<0.10$). Two of the five patients without abscess who had an increase in antibody concentration greater than 3.1 µg/ml had pathologic findings of pelvic inflammatory disease. Another of these five patients had a wound infection from which were cultured two types of anaerobic gram-negative bacilli which were not identified further.

Table 1. Comparison of Pre-Surgery and Discharge Serum Antibody Concentrations (in ug/ml) Among the Five Groups of Patients

| | No Complications | Wound Infection | Pelvic Cellulitis | Abscess | Fever |
|---|---|---|---|---|---|
| | (n=21) | (n=4) | (n=4) | (n=9) | (n=15) |
| Pre-Surgery | | | | | |
| Median | 5.2 | 4.1 | 4.3 | 5.6 | 5.6 |
| Range | 2.0 - 11.3 | 3.1 - 4.8 | 3.7 - 10.3 | 3.1 - 17.7 | 3.0 - 13.3 |
| Discharge | | | | | |
| Median | 6.0 | 5.5 | 5.5 | 13.0 | 7.8 |
| Range | 2.5 - 10.6 | 3.6 - 8.7 | 3.6 - 10.4 | 4.0 - 17.9 | 3.2 - 13.0 |

Table 2. Change in Concentration of Antibody to Capsular Polysaccharide Among the Five Groups of Patients

| No Complications | Wound Infection | Pelvic Cellulitis | Abscess | Fever |
|---|---|---|---|---|
| (n=21) | (n=4) | (n=4) | (n=9) | (n=15) |
| +4.7 | +4.3 | +1.6 | +12.4 | +4.4 |
| +2.1 | +3.3 | +0.9 | +8.9 | +3.7 |
| +1.6 | +0.8 | +0.1 | +7.4 | +2.8 |
| +1.6 | -0.8 | -0.1 | +3.9 | +2.6 |
| +1.5 | | | +3.8 | +1.4 |
| +1.5 | | | +3.7 | +1.3 |
| +1.1 | | | +3.6 | +1.3 |
| +1.1 | | | +0.3 | +0.8 |
| +0.9 | | | +0.2 | +0.4 |
| +0.7 | | | | +0.4 |
| +0.6 | | | | -0.3 |
| +0.6 | | | | -0.4 |
| +0.5 | | | | -0.8 |
| +0.5 | | | | -1.3 |
| +0.4 | | | | -1.9 |
| +0.3 | | | | |
| N.C. | | | | |
| -0.8 | | | | |
| -1.5 | | | | |
| -1.9 | | | | |
| -2.5 | | | | |
| $\bar{x}$= +0.62 | $\bar{x}$= +1.90 | $\bar{x}$= +0.63 | $\bar{x}$= +4.91 | $\bar{x}$= +0.95 |
| s.d.= 1.52 | s.d. 2.33 | s.d.= 0.78 | s.d.= 3.99 | s.d.= 1.81 |

## Discussion

Post-operative infections are the most important cause of morbidity following hysterectomy (10). The present investigation has considered this morbidity in terms of wound infection, pelvic cellulitis, abscess and fever. The data presented here suggest that the majority of abscesses, cuff or pelvic, are associated with an increase in antibody to the capsular polysaccharide of Bacteroides fragilis. The data further suggest that, with the possible exception of wound infections, the other infectious or presumably infectious complications of this procedure are uncommonly associated with such change in antibody concentrations.

These serologic data are consistent with bacteriologic findings of other investigators who have cultured genital abscesses in women. Thadepalli et al. cultured B. fragilis from 9 of 12 pelvic abscesses and from all of three tubo-ovarian abscesses in a series of thirty-three women with severe infection of the genital tract (3). Golde et al. isolated B. fragilis from 19 of 37 patients with pelvic abscesses which were cultured carefully for aerobes and anaerobes (5).

It is of interest to note that two patients with pathologic findings of pelvic inflammatory disease developed increases in antibody concentration of a magnitude similar to those observed in most patients in the abscess group, but had no evidence of abscess. In a study of 33 women with nongonococcal acute pelvic inflammatory disease, Eschenbach and associates found mixed aerobic and anaerobic bacterial peritoneal infection to be common. The most common species recovered were B. fragilis peptostreptococci, and peptococci (11). Those from whom B. fragilis was isolated from the cul-de-sac had significantly greater increases in antibody to the capsular polysaccharide of B. fragilis than those without B. fragilis isolated (7). Against this background, our observations suggest that surgical excision of adnexal tissue from women with chronic pelvic inflammatory disease may release or expose B. fragilis antigens which elicit a significant antibody response even in the absence of clinical infection.

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B. Chemical variation in capsular structure

In the process of preparing several lots of B. fragilis capsular polysaccharide for the vaccine safety and toxicity studies we found that lots differed considerably in terms of the specific monosaccharide constituents. Basically, 2 patterns were seen (Figure 1), one with several monosaccharide constituents (at least 4 and perhaps 5) and the other consisting primarily of glucose. Interestingly, both of these polysaccharides reacted with lines of identity with antisera prepared in rabbits to the more chemically complex antigen. The complex polysaccharide is composed of approximately 20% glucose, 20% galactose and at least 2 or 3 other sugars. Both these polysaccharides were extracted from cells grown in the same broth, for the same incubation period and were extracted by identical procedures (1). The extraction procedure consisted of purification of outer membrane after extraction with 0.01M EDTA and purification of the capsular substance from the membrane by procedures reported previously (1).

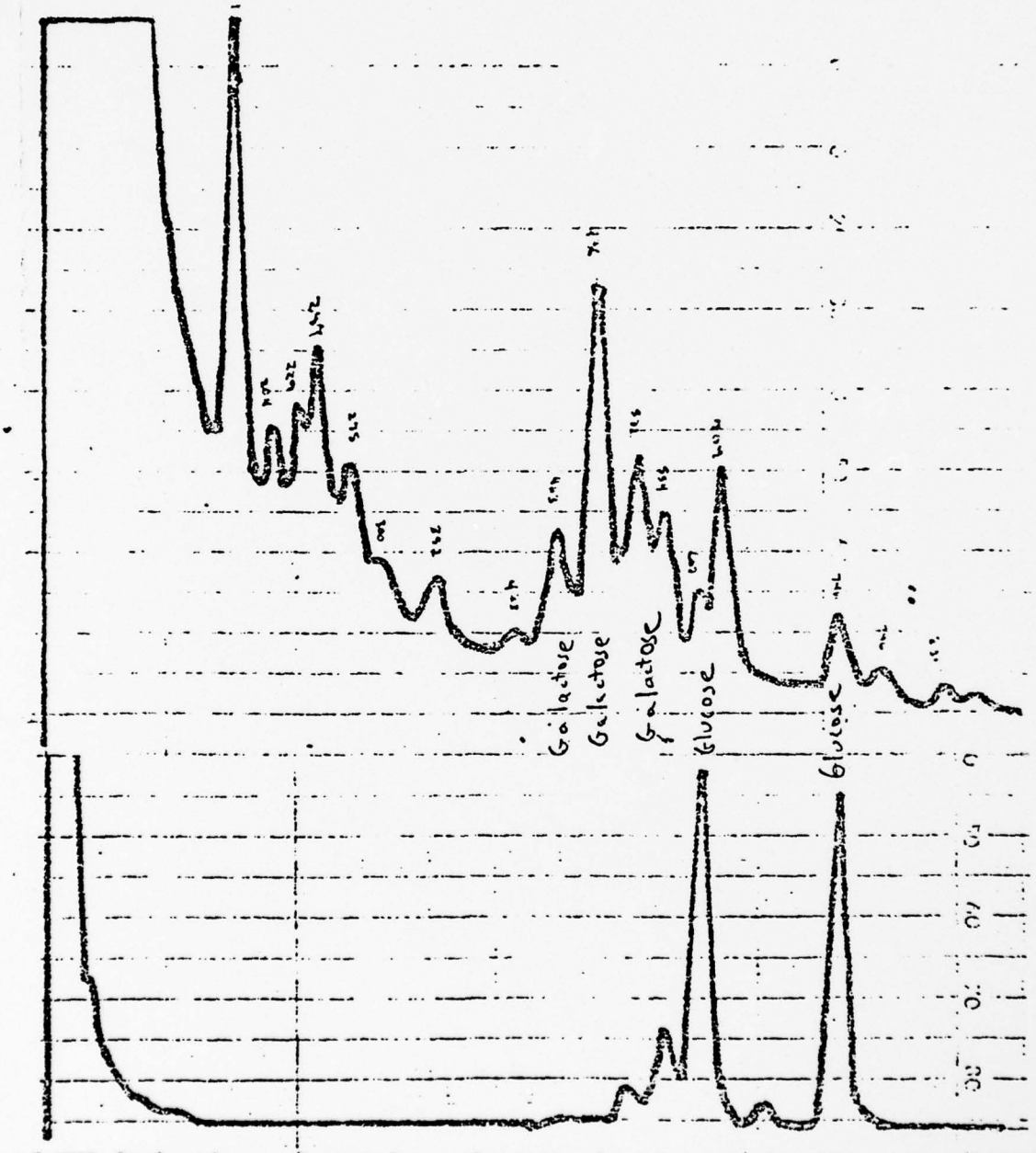
We noted that lot numbers of capsules prepared earlier in the year were all of the low glucose type while more recent lots were of the high glucose type.

We next went back to an original ATCC sample of strain 23745 and mass cultivated the organisms as outlined previously and extracted capsule. The capsule was purified and characterized and found to be of the low glucose type. We then did serial in vitro passages of this organism on blood agar plates and froze (at $-70^{0}$) organisms from each passage up through the 15th. We then prepared one large lot of media and grew in tandem organisms from the 3rd, 7th, 11th and 15th passage. Capsular polysaccharide was extracted from each culture using identical reagents and methods. We found that the 3rd and 7th passage capsule was of the low glucose type and the 11th and 15th passage capsule was of the high glucose type. We verified by biotyping the species identification of 20 colonies from each of these cultures and all were B. fragilis ss. fragilis. An obvious in vitro phenotypic change had taken place in capsular structure. To determine whether this was related to the culture media (blood agar plates) the high glucose antigen producing strain was re-implanted into a rat peritoneum in the animal model which we have previously described (2). After the formation of intraabdominal abscesses (one week after implantation) the B. fragilis strain was isolated from the abscess and the organism mass cultivated for capsule extraction. The resulting capsule was now of the low glucose type. Therefore, phenotypic reversion of capsular-structure took place after a single animal passage.

To determine if this phenotypic variation was limited to the capsular polysaccharide or whether other outer membrane associated antigens varied, the lipopolysaccharide and outer membrane protein of both the high and low glucose capsule producing strains were studied.

Lipopolysaccharide was purified as previously detailed, by separation from the outer membrane using detergents (3) and final purification on Sepharose 4B. The chemical composition of the LPS from the high and low glucose capsule strains were essentially identical (Table).

FIGURE 1




GLC profile of TMS-derivatives of capsular polysaccharide from B. fragilis strain 23745
top- low glucose capsule - isolated from strain on first passage from an abscess
bottom- high glucose antigen- isolated from same strain on 11th in vitro passage

| | LPS (low glucose capsule strain) | LPS (high glucose capsule strain) |
|---|---|---|
| Early Peaks | 7.5% | 11.3% |
| Galactose | 57.4% | 58.4% |
| Glucose | 19.6% | 11.49% |
| Glucosamin | 10.8% | 13.8% |

Outer membrane protein patterns were studied by polyacrylimide gel electrophoresis in SDS. The figure on the following page shows that the outer membrane protein patterns of the high and low glucose capsule variants of strain 23745 are essentially identical.

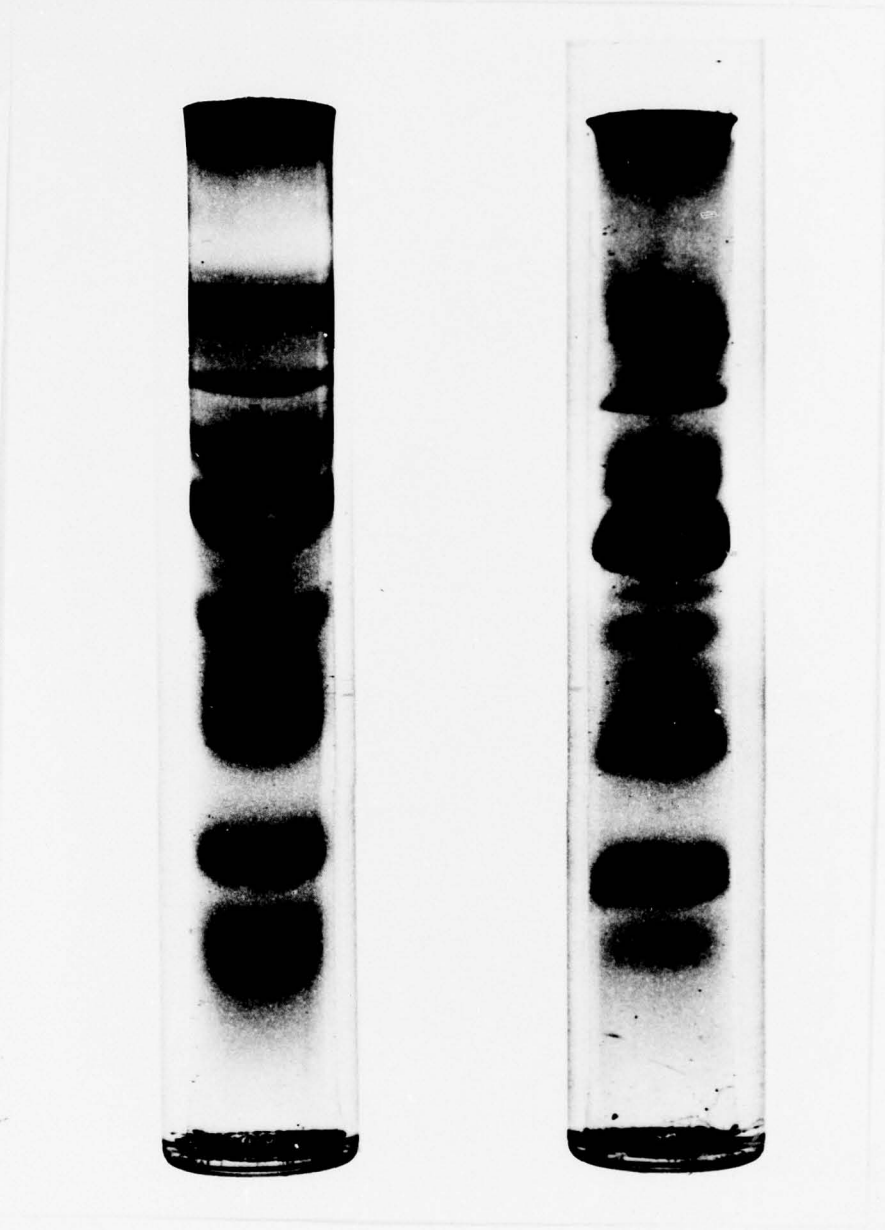


Figure SDS-PAGE of high and low glucose capsule variants of strain 23745, B. fragilis ss. fragilis.

Therefore, the phenotypic variation would appear to be only in capsular structure and not in lipopolysaccharide or outer membrane proteins.

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C. Assessment of the protective capacity of antibody to the B. fragilis capsular polysaccharide to the bacteremic phase of intraabdominal sepsis

Last year, we reported that parenteral immunizations of rats with the capsular polysaccharide of B. fragilis gave organism specific protection in our rat model (1) to the abscess phase of intraabdominal infections. We have now modified the model to test for the bacteremic phase.

Study Design:

Five week old male Wistar rats (160-180 grams), Simonson Laboratories (Palo Alto, California) were used for all experiments. Animals were housed separately and received Chow (Ralston-Purina, St. Louis, Missouri) and water ad lib diem.

After preimmunization sera were obtained, the animals were vaccinated three times a week for two weeks and given a booster in the third week. Four weeks after the initial vaccine was administered, the animals were infected intraperitoneally with various bacterial implants. Serum was also obtained at this time. One week later the animals were sacrificed and studied.

Immunization:

Animals were immunized with 10 µg B. fragilis capsular polysaccharide (0.1 ml) + 10 µg methylated bovine serum albumin (0.1 ml) + 0.2 ml complete Freund's adjuvent, IM, at the onset of the experiment and on the ninth day after immunization was started. On the remainder of the vaccination days, these animals received 10 µg B. fragilis capsular polysaccharide (0.1 ml) with 10 µg methylated bovine serum albumin (0.1 ml).

Non-immunized control groups were subjected to an identical routine, but received injections of PBS, MBSA and Freunds adjuvent without capsular polysaccharide.

Sera:

Aliquots of blood (0.5 ml) were obtained from each rat by percutaneous transthoracic cardiac puncture prior to implantation and at the designated intervals thereafter. Serum was separated from the clotted blood by centrifugation at 2,000 g for 15 minutes and stored frozen at -70°C.

Challenge:

Preparation of challenge bacteria: Quantitative determinations of viable cell density were obtained for all bacterial strains after quick-freezing of the original culture. Aliquots of each species were thawed within an anaerobic chamber, and serial 10-fold dilutions were made with use of VPI dilution salts. Samples (0.1 ml) of each dilution were plated on prereduced brucella-base blood agar. Plates were incubated for 48 hours at 37°C within the anaerobic chamber, and colonies were counted. Viable cell density was expressed as cfu/ml. Before implantation bacterial cultures were diluted to the appropriate concentration with sterile PYG broth. The number of bacteria implanted were expressed as viable cfu per implant, and have been reported previously (1).

Innocula:

The two groups received challenge inocula consisting of a combination of enterococci and B. fragilis. The challenges given with 10% (wt/vol) $BaSO_4$, 50% (vol/vol) sterile cecal contents and the challenge bacteria. Control experiments have shown that animals receiving $BaSO_4$ and sterile cecal contents do not develop abscesses (1).

Animals:

After completion of the immunization schedule, animals were anesthetized by intraperitoneal injection of .15 ml of nembutal (50 mg/ml, Abbot Labs, North Chicago, Illinois) and the abdomens were shaved and swabbed with tincture of iodine. A 1 cm anterior midline incision was made through the abdominal wall and peritoneum and a double gelatin capsule containing 0.5 ml of the innoculum was inserted into the pelvic region. The incisions were closed with interrupted 3-0silk sutures and the animals were returned to separate cages. The surgical mortality rate in all groups was less than 1% of animals with implants.

Blood cultures were obtained by percutaneous transthoracic cardiac puncture. 0.1 ml aliquots were immediately placed into 19 ml of molten brain heart infusion and poured into sterile petri plates. A second 0.1 ml aliquot was diluted 100-fold in sterile VPI dilution salts and 0.1 ml of this was plated in an identical manner. All plates were incubated at 35°C in an anaerobic chamber for 3-5 days. Colonies were enumerated and representative types isolated and identified by established criterion.

Results:

The ability of the vaccine to prevent Bacteroides fragilis bacteremia was studied in 10 immunized animals, and the results compared to 10 controls. The median preimmunization antibody concentration in this group was <1.5 µg/ml and after immunization was 10.2 µg/ml. Animals were challenged with B. fragilis (23745) given intraperitoneally as outlined above and serial blood cultures were randomly sampled from five of these animals at various times after challenge. Only five of the 10 were sampled at each time interval because of sporadically occurring deaths due to the transthoracic sampling of blood. None of the immunized animals became bacteremic after intraperitoneal implantation of B. fragilis. All the non-immunized animals became bacteremic within one hour after implantation with mean $\log_{10}$ CFU/ml 3.57 ± .17. Therefore, immunization with the B. fragilis capsule prevents bacteremia as well as abscess formation in this animal model due to B. fragilis (Table).

We believe that these experimental data offers strong support to the feasibility of immunization of humans to prevent B. fragilis infection.

Results of quantitative blood cultures

| | Hours after intraperitoneal challenge with B. fragilis | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 24 | 48 | 96 |
| immunized<br>no. +/total (mean $\log_{10}$ CFU/ml | 0/5 ($<10^1$) | 0/5 ($<10^1$) | 0/5 | 0/5 | 0/5 |
| controls<br>no. +/total (mean $\log_{10}$ CFU/ml | 5/5<br>(3.57 ± .17) | 5/5<br>(3.6 ± .22) | 2/5 | 0/5 | 0/5 |

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D. Passive Protection Studies

High titered rabbit antiserum was prepared to the capsular polysaccharide of B. fragilis by methods previously described (1). This serum was demonstrated to be high in antibody titer using the RABA (2). Similarly, high titered rabbit serum to the Re mutant of S. Minnesota was obtained from Dr. Stephen Zinner at Brown University. 0.5 µl of each serum was given to separate groups of rats prior to and at 4 hours after intraperitoneal challenge {as outlined above and previously (3)} with B. fragilis and E. coli. The sera were given to rats either intravenously or intramuscularly. No protective effect was seen in passively immunized animals to either the peritonitis or abscess phase of experimental intraabdominal sepsis.

The failure of high titered anti B. fragilis and Re rabbit serum to protect rats from intraabdominal infection contrasted with the ability of active immunization with B. fragilis capsule to protect against B. fragilis infection presents an interesting problem. It may simply be that passive protection is not a satisfactory method of preventing B. fragilis infection, however, this really should not be the case if active immunization works as well as it seems, it may be that the heterologous rabbit serum is not adequately activating rat complement, or the rabbit antibody may be binding to rat antibody forming immune complexes. In either case the experiments need to be done using rat serum in passive protection studies of rats. (See renewal proposal)

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E. Development of an animal model of wound abscess

Previously summarized studies indicate that rats challenged intraperitoneally with an inoculum of cecal contents developed an infection which simulated intra-abdominal sepsis as it is encountered in clinical practice according to both bacteriological and pathological criteria. This work indicates that both coliforms and anaerobes are pathogens in this model. However, these organisms appear to have distinctive roles in the pathogenic events as the infection evolves from generalized peritonitis to abscess formation. Of particular interest are the studies with B. fragilis which show that pure cultures of encapsulated strains of this organism produce intraabdominal abscesses. This work forms the background for our more recent studies which will be described in greater detail.

An animal model simulating human wound infection was developed since anaerobic bacteria are often involved in these infections and the host defenses in the subcutaneous tissues may be considerably different than those present in the peritoneal cavity. In addition, a subcutaneous infection would allow daily inspection of developing lesions.

Male Wistar rats weighing 200-250 grams were used for all experiments. Animals were anesthetized with 0.15 ml of nembutol (50 μg/ml) and the back was shaved and prepped with iodine. A 1 cm incision through the skin and underlying fascia was made on either side of the midline approximately 1 cm from the thoracic spine. The incisions were closed with two sutures using 3-0 silk. Inocula consisting of either broth cultures alone, cecal contents or sterile cecal contents plus bacterial cultures were injected through the closed incision using an 18 gauge needle. Details for preparation of inocula have been described in previous reports. Aliquots of 0.25 ml of each inoculum were injected through each incision.

Wounds were examined daily for evidence of infection. Each wound was cultured at seven day intervals using needle aspirates of 0.05ml. Blood cultures were obtained from animals as described previously and bacterial strains isolated from blood or wounds were identified by established criteria.

Initial experiments employed the subcutaneous injection of the cecal inoculum and barium sulfate into rats directly beneath a surgical incision. It was found that animals implanted in this manner developed discrete abscesses within 5 days which became progressively larger until the 14th to 17th day (Table 1).

Table 1

RESULTS OF IMPLANTING VARIOUS INOCULA
INTO RATS THROUGH A SKIN INCISION

| Inoculum | Mortality | Abscess | Positive Blood culture |
|---|---|---|---|
| Cecal contents + $BaSO_4$ | 0/10* | 10/10** | 1/10 (E. coli) |
| Cecal Contents | 0/10 | 10/10 | 0/10 |
| Broth cultures of: | | | |
| B. fragilis | 0/10 | 0/10 | 0/10 |
| B. melaninogenicus | 0/10 | 0/10 | 0/10 |
| E. coli | 0/5 | 0/5 | 0/5 |

* No. died/no. tested
** No. with abscess/no. tested

The mean Eh of the purulent material measured *in situ* at 7 days was -113 mv in six animals tested, indicating the presence of a reduced environment. The abscess drained spontaneously through the site of the incision between the 14th to 17th day after challenge and, in some cases, drainage occurred through sinus tracts 2 to 3 cm from the incision. In contrast to the previously described intraabdominal sepsis model, no mortality was noted in these animals. Blood cultures at 24 hours were positive in only 1 of 10 animals, with *E. coli* being the single bacterial isolate. Cultures of abscess pus were uniformly positive for *E. coli*, enterococci and *B. fragilis*. Another difference compared to the intraabdominal sepsis model was the observation that cecal contents implanted without barium sulfate produced abscesses in all ten recipients.

Implants composed of pure cultures of *B. fragilis* (ATCC-23745), *B. melaninogenicus* (109-6), and *E. coli* (BVA 1-13), each at a concentration of $5X10^7$ CFU/ml, failed to cause abscesses in the soft tissue infection model. However, implants of the same inoculum size of *B. fragilis* combined with 50% sterile cecal contents resulted in abscess formation in all recipients.

Table 2

RESULTS OF IMPLANTING VARIOUS BACTEROIDES SPECIES INTO RATS THROUGH A SKIN INCISION

| Inoculum* | Mortality | Abscess | (mm)** |
|---|---|---|---|
| *B. fragilis* | 0/15 | 15/15 | (6.0) |
| *B. fragilis* after immunization | 0/5 | 5/5 | (6.0) |
| *B. distasonis* | 0/5 | 0/5 | |
| *B. thetaiotaomicron* | 0/5 | 3/5 | (3.2) |
| *B. vulgatus* | 0/5 | 3/5 | (4.0) |
| *B. assaccharolyticus* | 0/15 | 15/15 | |
| *B. melaninogenicus* ss. *intermedius* | 0/10 | 10/10 | |

* All cultures contain $5X10^7$ CFU and 50% sterile cecal contents

** No. positive/no. tested (mean abscess diameter)

Interestingly, animals immunized with the capsular polysaccharide from the homologous strain of *B. fragilis* prior to implantation (1) also developed abscesses. These data suggest that circulating antibody has little ability to prevent abscess formation or to eliminate *B. fragilis* from the abscess during wound infection. Implants of other closely related *Bacteroides* sp. combined with 50% sterile cecal contents showed abscess formation in 6 of 15 animals.

Additional studies have been performed using inocula composed of 50% sterile cecal contents combined with *B. assaccharolyticus* (382,536). This organism was of

particular interest in view of recent studies showing the presence of a capsular polysaccharide (2). All fifteen animals implanted with this inoculum developed abscesses. Additionally, all ten animals implanted with B. melaninogenicus ss. intermedius (10946, BVA 1-21), a phenotypically similar species, also developed abscesses. These data indicate that a number of Bacteroides species have the ability to potentiate subcutaneous abscess in this model. This model was of interest because of the possibility that the host defenses during soft tissue infection are considerably different than those found in the peritoneum. It was found that cecal contents with or without the inclusion of barium sulfate caused abscess when implanted subcutaneously in Wistar rats. In contrast to the intraperitoneal infection with the same inoculum, no mortality was noted and bacteremia was rarely detected. Additionally, the adjuvent effect of barium sulfate was not required for abscess formation. These results indicate that the host response to the same inoculum was dependent to some extent on the site of the infection.

These observations are also supported by the finding that animals immunized with the capsular polysaccharide of B. fragilis developed subcutaneous abscesses when challenged with the homologous strain of this organism. Previous research has shown this immunization protects against abscess formation when animals are challenged with B. fragilis introduced intraperitoneally. Additionally, a comparison of the ability of B. fragilis and related species to cause abscess showed that unencapsulated strains of organisms previously classified as B. fragilis caused abscesses in some animals challenged subcutaneously, but not intraperitoneally. Subcutaneous challenge of another encapsulated Bacteroides sp., B. assaccharolyticus, also resulted in abscess formation. Interestingly, a closely related species, B. melaninogenicus ss. intermedius also produced abscesses. These results suggest that one criteria for virulence among the Bacteroides, appears to be a capsular polysaccharide although the unique composition of the LPS of other organisms may play a significant role in their pathogenicity.

The information provided in the present report point out several important features of animal models. The most essential feature of any animal model is the similarity of animal infection to human disease. Previous researchers have reported the ability of obligate anaerobes alone and in conjunction with other facultative bacterial species to cause disease in animals. These investigations suggested a significant pathogenic role for anaerobes, but clinical utility of the data was limited by the failure of the models to simulate human disease. An attractive feature of the model described here is that it simulated intraabdominal sepsis as it is encountered clinically by both pathologic and bacteriological parameters.

A final consideration essential to evaluation of animal models for human infection is the host response to bacterial challenge. The ability of an animal host to respond in a particular manner to infectious insult is governed in large part by the animals' own immune response. The similarity of animal and human humoral and cell mediated immunity are necessary important considerations. Additionally, the host response to challenge may vary depending on the site of the infectious focus. This point has been demonstrated experimentally by comparing host response to intraperitoneal and subcutaneous challenge with an identical inoculum in the Wistar rat. The finding that mortality and relative virulence of certain species was different suggests that single animal models cannot be used to draw conclusions about bacteriologically similar lesions in different tissues and demonstrates the organotropism of certain pathogenic species. Intraabdominal abscesses and subcutaneous abscesses both harbored a microflora which was identical, yet the development of the lesions, the role of specific species in the infection, and the role of circulating antibody were different. These data suggest that in order to apply the lessons learned from animal models to human disease, the site of infection must be similar.

Despite the wealth of emperical and experimental information available on bacterial infections of humans, animal models remain an important [illegible] elucidating the etiology

of certain infections and in identifying the pathogenic factors associated with the infection microflora. The judicious use of animal models continues to increase our understanding of the infectious process and often provides data not readily available through the study of human subjects.

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F. Bacteroides thetaiotaomicron (B. fragilis ss. thetaiotaomicron)

Immunochemical studies are in progress on the outer membrane of B. thetaiotaomicron. Although clinically, B. fragilis ss. fragilis is the most important overall isolate of the B. fragilis group, we have found B. thetaiotaomicron to be next in importance (Table), by our continuing surveillance of clinical isolates.

ISOLATION RATES OF ORGANISMS PREVIOUS CLASSIFIED AS BACTEROIDES FRAGILIS IN CLINICAL SPECIMENS

| Organism according to current taxonomic schema | Blood culture (56 patients) | Exudate culture (227 patients) |
|---|---|---|
| B. fragilis | 43 (77%) | 144 (63%) |
| B. thetaiotaomicron | 4 (7%) | 53 (23%) |
| B. distasonis | 5 (9%) | 26 (11%) |
| B. vulgatus | 2 (2%) | 26 (11%) |
| B. ovatus | 1 (2%) | 24 (11%) |
| Bacteroides "other"* | 1 (2%) | 18 (8%) |

* These organisms were previously classified as B. fragilis, but failed to conform to criteria for subspeciation.

We have purified and partially characterized outer membrane and outer membrane constituents from 17 strains of B. thetaiotaomicron. These studies have been designed to look for common antigens within this species or perhaps antigens which are shared with B. fragilis. The reason for this latter possibility is that we have found that a few strains of B. thetaiotaomicron react in our IFA (indirect fluorescent antibody) test with B. fragilis antiserum.

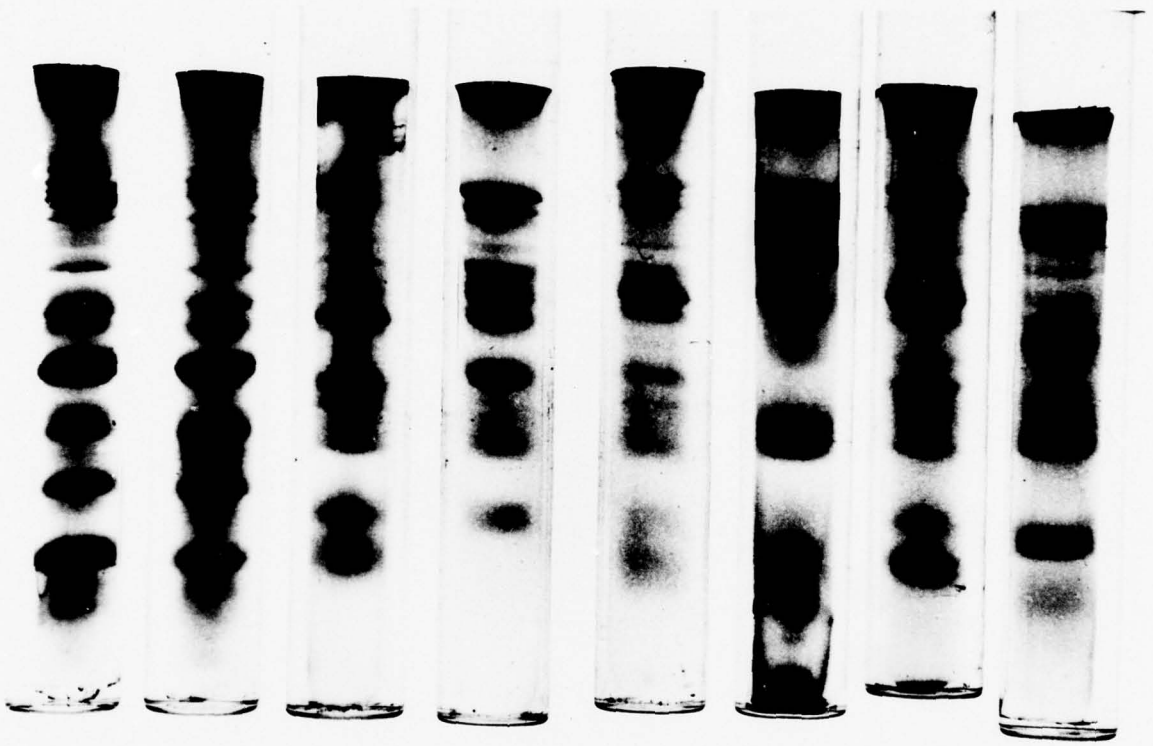
Preliminary chemical analyses of B. thetaiotaomicron outer membranes and outer membrane constituents after gel chromatography on Sephadex G-100 in 0.5% sodium deoxycholate buffer (1) are listed in the following Table. The chromatographic profiles of the outer membranes of this species are identical to those reported previously for B. fragilis and we will not review that here (1).

| Strain (all clinical isolates) | Outer Membrane %protein (2) | % carbohydrate (3) | Peak 1 % protein | %CHO | LPS (peak 2) % prot. | % CHO |
|---|---|---|---|---|---|---|
| 2327 | 85 | <10 | - | - | <2% | - |
| 2410 | 73 | 11 | 88 | 11 | <2% | 11.9 |
| 4085-10 | 41 | 32 | 53 | 37 | <2% | 18.2 |
| 8492 | 24 | 20 | 43 | 40 | <2% | 19.7 |
| 12290 | 91 | <10 | 96 | 10 | <2% | 20.5 |
| 20230-3 | - | - | 29 | 10.5 | <2% | 14.2 |
| 20649-4 | 83 | 15 | 71 | 18 | <2% | 15 |
| 23356 | - | - | 81 | 6 | <2% | 13.7 |
| 23839 | 70 | <10 | - | - | <2% | - |
| 24390 | 26 | 42 | 25 | 47 | <2% | 19.2 |
| 28899 | 78 | 25 | 76 | 25 | <2% | 15.7 |
| 29415 | 58 | <10 | 80 | 10 | <2% | 26 |
| 40525 | 89 | <10 | 73 | 16 | <2% | 10.5 |
| 41113 | 34 | 34 | 35 | 46 | <2% | 26 |
| 43172 | 43 | 14 | 56 | 17 | <2% | 16 |
| 73038 | - | - | 89 | 71 | <2% | 19 |
| 73996 | 79.5 | <10 | 62 | 15 | <2% | - |

These chemical analyses do indicate that perhaps at least some strains have chemical evidence of capsules (as defined by a high carbohydrate content in peak 1). This is not in the least bit definitive however. The material must be isolated; purified and characterized. This capsular polysaccharide, should it exist, would not appear to be common to all strains, because several strains have very little carbohydrate in peak 1.

The carbohydrate in peak 1 may be non-disaggregated LPS or it may be a result of interference in the phenol-sulfuric acid assay from the high protein content of peak 1.

SDS-polyacrylimide gel electrophoresis, utilizing the method of Weber and Osborn (4) of these B. thetaiotaomicron outer membranes was done. Interestingly, one strain which cross reacted with the B. fragilis serum (73996) had an identical outer membrane protein pattern to B. fragilis (Figure 1). However, strains of B. thetaiotaomicron in general have outer membrane protein band patterns distinct from each other and from other B. fragilis strains, a finding which distinquishes them from B. fragilis strains which share identical outer membrane proteins by this technique (1).



Legend - SDS-PAGE of outer membranes of Bacteroides species. B. fragilis 23745, B. thetaiotaomicron 73996, 29414, 4057, 2410, 20230-2, 28899, 41113.

Chemical analyses of the lipopolysaccharides of 4 strains of B. thetaiotaomicron is shown in the Table below. Like B. fragilis strains, these strains have no KDO or heptose in the lipopolysaccharide. There is, however, considerable strain variability in the carbohydrate comparison of the LPS.

Chemical composition of the LPS of B. thetaiotaomicron strains

| | 20230-2 | 28899 | 29415 | 40575 |
|---|---|---|---|---|
| Early Peaks (unidentified) | 2% | 46% | 33 | 80 |
| Mannose | 7 | 9 | 8 | 2 |
| Galactose | 16 | 28 | 19 | 3 |
| Glucose | 70 | 7 | 38 | 4 |
| Glucoseamine | + | 8 | + | 1 |
| KDO | none | none | none | none |
| Heptose | none | none | none | none |

Immunologic studies of B. thetaiotaomicron.

Antisera were prepared on rabbits to 6 strains of B. thetaiotaomicron. These sera were prepared to viable organisms using multiple injections over a 1 month period, as outlined for B. fragilis previously (1).

Double diffusion in agar studies (5) using the purified outer membrane fractions of B. thetaiotaomicron strains against these sera showed very minimal cross reactivity. Most strains gave precipitin lines with homologous LPS fractions and gave no precipitins with peak 1 fractions. This indicated that unlike B. fragilis, a B. thetaiotaomicron common species specific antigen is not readily apparent. Because of the reactivity in these analyses was with the LPS fraction, indirect hemagglutinatation (6) was used to study whether there were common LPS determinants which were not apparent by double diffusion. Sheep erythrocytes were sensitized with LPS from various strains of B. thetaiotaomicron and these were tested on IHA for cross reactivity with the B. thetaiotaomicron sera, (Table on the next page). Some rather broadly reactive LPS antigens were found, for example LPS from strains 2410 and 40575. On the other hand some LPS antigens were poorly reactive (28899).

Geometric Rise in HA titer

| | | Antisera to Bacteroides strains | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LPS from strain No. | B. thetaiotaomicron 2410 | 40575 | 29415 | 23839 | 12290 | 41113 | B. fragilis 23745 |
| B. thetaiotaomicron: | 73038 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| " " " | 8492* | 0 | 1 | 0 | 1 | 2 | 1 | 0 |
| " " " | 20649-4* | 0 | 1 | 0 | 2 | 2 | 1 | 1 |
| " " " | 29415* | 0 | 0 | 1 | 0 | 0 | 1 | 1 |
| " " " | 28899 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| " " " | 24390 | 0 | 0 | 0 | 1 | 0 | 1 | 3 |
| " " " | 20230-2 | 2 | 1 | 0 | 2 | 1 | 0 | 0 |
| " " " | 23356 | 1 | 1 | 0 | 1 | 3 | 0 | 0 |
| " " " | 4085-10 | 1 | 0 | 1 | 5 | 3 | 0 | - |
| " " " | 41113 | 2 | 2 | 1 | 3 | 3 | 2 | - |
| " " " | 12290 | 2 | 1 | 2 | 4 | 2 | 0 | - |
| " " " | 2410 | 1 | 3 | 2 | 4 | 2 | 1 | - |
| " " " | 40575 | 3 | 2 | 1 | 3 | 3 | 2 | - |
| " " " | 43172 | 2 | 0 | 0 | 2 | 1 | 1 | - |
| B. fragilis: | 23745 | 0 | 0 | 0 | 1 | 1 | 0 | - |

Therefore, at least some strains have rather broadly reactive LPS molecules. The LPS of the Bacteroides species are essentially non-endotoxic in experimental animals (7), therefore, these broadly reactive LPS molecules may be good candidates for further study in the development of vaccines.

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## II. Bacteroides Melaninogenicus

### A. Background

We have isolated and partially characterized the antigens of Bacteroides melaninogenicus subspecies asaccharolyticus that comprise the surface, or outer membrane of the organism (1). Two major antigenic components were found.

The first is a protein-polysaccharide-loose lipid complex. Purification of the polysaccharide component of this fraction revealed a molecular size of $7.2 \times 10^5$ daltons which serologically cross-reacted with similarly-isolated polysaccharides from other strains of this subspecies. Preliminary work suggested that this surface antigen was a polysaccharide capsule.

The second major outer membrane fraction of B. melaninogenicus subspecies asaccharolyticus was demonstrated to be a lipopolysaccharide (2). Curiously, the lipopolysaccharide contains a core structure that is atypical of the LPS of facultative gram-negative bacteria in that it lacks two of the common core sugars, keto-deoxyoctonate (KDO) and heptose, as well as the most common fatty acid constituent of the Lipid A moiety, B-hydroxymyristic acid. This chemical aberrancy may explain partially the further observation that this LPS material is biologically inactive relative to LPS of aerobic gram-negative bacteria.

### B. Review of Progress: Immunochemical studies of the capsular polysaccharide of Bacteroides melaninogenicus subspecies asaccharolyticus

1. Identification of a subspecies specific capsular antigen by electron microscopy and immunofluorescence.

   a. Materials and Methods

      Bacterial strains

Bacteroides melaninogenicus strains isolated from an oral source (gingival crevice) were obtained from Dr. A. Crawford, Forsyth Dental Laboratory, Boston, Massachusetts. Strain 18946 and 73044, isolated from lung abscesses, were obtained from Ms. K. Daly, Boston City Hospital. Other non-oral isolates of B. melaninogenicus were provided by Dr. A. Onderdonk, Boston V.A. Hospital. Strains of Bacteroides fragilis, B. thetaiotaomicron, B. vulgatus, and B. distasonis were clinical isolates from our collection. Strains of Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa were obtained from infected urine and provided by Ms. Mary Kendrick, Channing Laboratory. Streptococcus pneumoniae, types 19 and 26, were provided by Dr. M. Grizzard, Channing Laboratory.

All Bacteroides isolates were stored at -70° C in peptone-yeast broth (Scott Laboratories, Fiskeville, Rhode Island) and were grown on blood agar supplemented with manadione (0.5 µg/ml) in an anaerobic jar (Gaspak, Baltimore Biological Equipment, Baltimore, Maryland) prior to use. The facultative gram-negative strains were taken from blood agar plates soon after clinical isolation. S. pneumoniae strains were stored frozen and cultured on blood agar plates in a reduced oxygen environment (candle jar)

at 37° C prior to use.

Bacteroides and Fusobacterium isolates were subspeciated according to the criteria outlined in the Anaerobic Manual (3). E. Coli, Klebsiella, Proteus, and Pseudomonas were identified by their fermentation pattern by means of the API method (4).

Antiserum

Capsular polysaccharide was purified from the outer membrane complex of strain 381 (Bacteroides melaninogenicus subspecies asaccharolyticus) as described previously (1). Purified capsular polysaccharide was used to immunize a 2 kg. New Zealand white rabbit. Capsular polysaccharide was suspended in phosphate-buffered saline (PBS), pH 7.4, to give a concentration of 0.5 mg/ml. 0.1 ml (50 µg) was injected intravenously three times per week for two weeks. A booster dose was given during the third week. The rabbit was bled one week later. In addition, capsular polysaccharide (50 µg) was mixed with 50 µg methylated bovine serum albumin (MBSA) and given with Freund's complete adjuvant, 0.25 ml, in the hind footpad once per week for the three weeks of immunization. Serum was stored at -70° C prior to use. Control serum was obtained from the same animal prior to immunization.

Preparation of Slides for Immunofluorescence

Glass slides were prepared as follows: Eight wells were made on each slide by dotting the slide with tiny drops of glycerol. The slide was then sprayed lightly with teflon (Fluoroglide; Chemplast, Inc., Wayne, New Jersey). The glycerol was rinsed off with water and the slide dried; wells were located where the glycerol had been).

Bacteroides and Fusobacterium strains were grown anaerobically overnight in peptone-yeast broth (Scott Laboratories) at 37° C. Facultative gram-negative strains of pneumococci were grown overnight at 37° C in Mueller Hinton broth. Purity was confirmed by gram stain and culture. The organisms were pelleted at 8000 g x 10 min and washed three times with 0.9% NaCl. The pellet was then suspended in 0.5 ml of PBS. Two µl were then suspended in 0.6 ml PBS. One µl of this suspension was placed on each of six wells on a slide and air dried. In all tests, strain 381 (Bacteroides melaninogenicus subspecies asaccharolyticus) was placed in a similar dilution on the two end wells of each slide for use as positive and negative controls. After drying, the slides were gently heat-fixed, then rinsed in distilled water to remove precipitated salts.

Rabbit antiserum prepared against capsular polysaccharide (from strain 381) was then added in 5 µl quantities to each of three wells in the top row at 1:50, 1:100, and 1:200 dilution after this range was determined to be optimal for the tests. Control serum at these same dilutions was added to the corresponding wells in the lower row. In the pair of end wells containing the homologous strain, immune serum (1:200) and control serum (1:200) were placed to provide a positive and negative control (with strain 381) for each slide. Slides were incubated in a moist chamber at room temperature for 30 min, washed with PBS for 10 min, rinsed in distilled water, and air dried.

Fluorescein-conjugated IgG fraction of goat anti-rabbit globulin (Cappel Laboratories, Downington, Pennsylvania) was diluted 1:8. Two µl were added to each well. The slides were incubated again at room temperature for 30 min, washed for 10 min with PBS, rinsed with distilled water, and air dried. One drop of glycerol-carbonate buffer mounting fluid, (0.05 M sodium carbonate buffer, pH 9.0, and glycerol, 1:9) was placed on each well and covered with a coverslip.

Slides were read under oil-immersion (100 x) with a Zeiss Fluorescent microscope (Carl Zeiss, New York, N.Y.) and a mercury HBO-200 W bulb. A heat-absorption light source (BG-12) and a 500 nm exclusion filter were used. Fluorescence was graded 0 to 4+ by two observers. Strains with 3+ or 4+ fluorescence were considered positive; strains with 0, 1+ or 2+ fluorescence were deemed negative. Each strain was graded with respect to a control at the same dilution; a positive and negative control were included on each slide for comparison.

Electron microscopy

Bacteria were grown overnight in trypticase yeast extract basal medium (1) and were prepared for electron microscopy after staining with ruthenium red to visualize the capsular polysaccharide as detailed previously (5).

## Results

### Indirect fluorescent antibody test (IFA)

A previous study has demonstrated the presence of a capsular polysaccharide as part of the antigenic structure of the outer membrane complex of Bacteroides melaninogenicus subspecies asaccharolyticus (1). In Table 1 the results of IFA studies performed against a number of bacterial species using rabbit antiserum prepared specifically to this capsular antigen are detailed.

Table 1: Results of indirect fluorescent antibody (IFA) test using anti-capsular antibody from Bacteroides melaninogenicus subspecies asaccharolyticus

| Bacterial species | Positive* | Negative† |
|---|---|---|
| Bacteroides melaninogenicus ss. asaccharolyticus | 23 | 0 |
| B. melaninogenicus ss. intermedius | 0 | 11 |
| B. melaninogenicus ss. melaninogenicus | 0 | 2 |
| Bacteroides fragilis | 0 | 4 |
| Bacteroides distasonis | 0 | 2 |
| Bacteroides vulgatus | 0 | 1 |
| Bacteroides thetaiotaomicron | 0 | 2 |

| Bacterial species | Positive* | Negative† |
|---|---|---|
| Fusobacterium nucleatum | 0 | 9 |
| Escherichia coli | 0 | 7 |
| Klebsiella pneumoniae | 0 | 1 |
| Pseudomonas aeruginosa | 0 | 2 |
| Proteus mirabilis | 0 | 1 |
| Streptococcus pneumoniae | 0 | 2 |

*3-4+ fluorescence was considered positive
†0-2+ fluorescence was considered negative. Two strains of B. melaninogenicus ss. intermedius had 2+ fluorescence. All other negative strains were 0 or 1+ fluorescence.

Twenty-three strains of Bacteroides melaninogenicus subspecies asaccharolyticus were studied by IFA and all were found to be fluorescent-positive, indicating that these strains all contained an antigenically similar determinant to that of the capsule of strain 381. Eleven strains of B. melaninogenicus ss. intermedius and two strains of B. melaninogenicus ss. melaninogenicus were fluorescent-negative, indicating that these strains lacked the capsular determinant of strain 381. Similarly, all other bacterial species tested were also fluorescent negative. These included 9 strains of the B. fragilis group, 9 strains of F. nucleatum, 11 facultative gram-negative bacterial strains, and 2 strains of S. pneumoniae. The fluorescent labeling of a cross-reactive capsular antigen was specific for strains of Bacteroides melaninogenicus ss. asaccharolyticus. Figure 1 demonstrates the intense (4+) fluorescent staining of the capsule of B. melaninogenicus ss. asaccharolyticus. Minimal reactivity (1-2+) was noted in two strains of B. melaninogenicus ss. intermedius, but virtually all strains were distinctly negative.

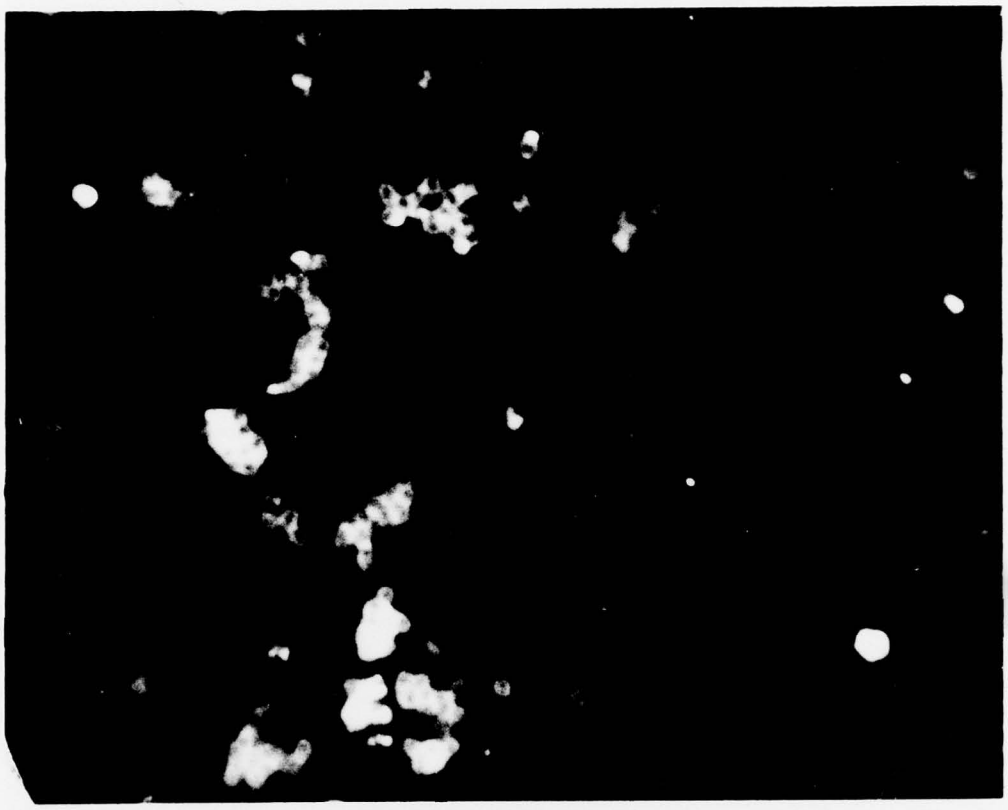

Figure 1. Fluorescent photomicrograph of a strain of Bacteroides melaninogenicus subspecies asaccharolyticus labeled by the IFA technique with rabbit serum to capsular antigen of a strain (381) of the same subspecies. The capsule shows intense (4+) fluorescence. Strains considered to be negative (0-2+ fluorescence) could not be seen clearly by photography at a similar exposure. Magnification x 2000.

Morphologic study of the capsule by electron microscopy

The presence and morphology of a capsular polysaccharide of B. melaninogenicus was defined by electron microscopy. Figure 2 demonstrates by ruthenium red staining the presence of a capsule external to the outer membrane of three strains of Bacteroides melaninogenicus ss. asaccharolyticus.

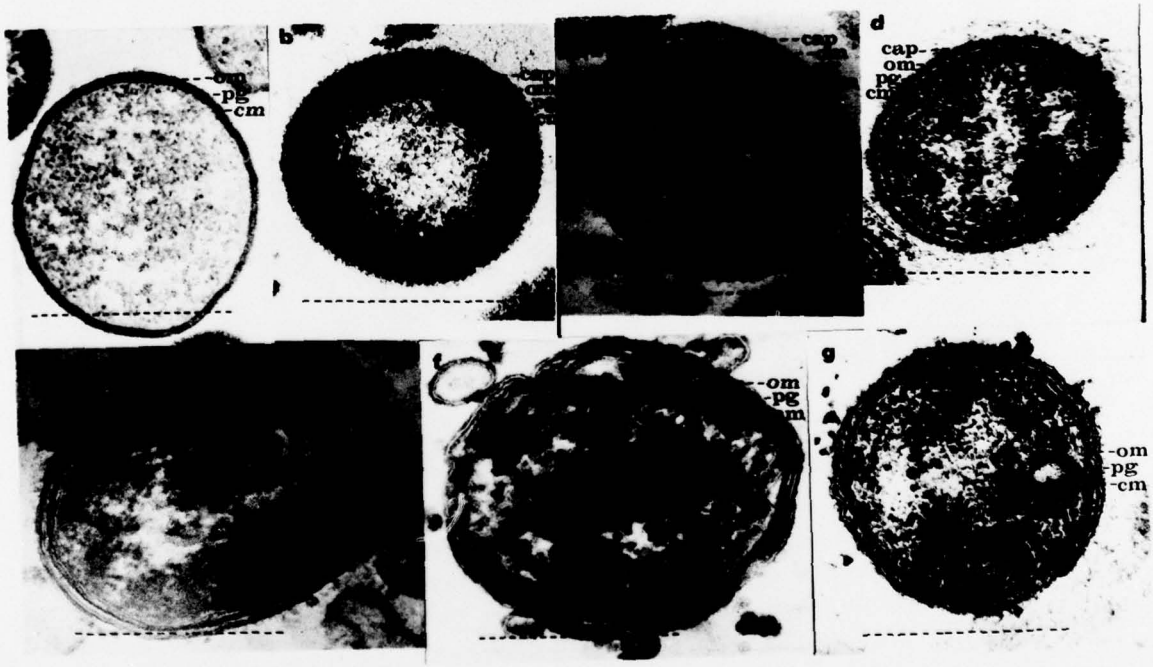

Figure 2. Electron micrographs of thin sections of Bacteroides melaninogenicus. 2a - B. melaninogenicus ss. asaccharolyticus without ruthenium red staining. 2b - B. melaninogenicus ss. asaccharolyticus, strain 376, stained with ruthenium red; 2c - B. melaninogenicus ss. asaccharolyticus, strain 382, stained with ruthenium red; 2d - B. melaninogenicus ss. asaccharolyticus, strain 536 stained with ruthenium red; 2e - B. melaninogenicus ss. intermedius, strain 576, stained with ruthenium red; 2f - B. melaninogenicus ss. intermedius, strain 581, stained with ruthenium red; 2g - B. melaninogenicus ss. melaninogenicus strain 379, stained with ruthenium red. cm = cytoplasmic membrane; pg - peptidoglycan; om - outer membrane; cap = capsule. Dashed line denotes 1 μm. Magnification x 75,000.

Two morphologic variations were noted. Most of the encapsulated strains showed a dense staining of variable thickness (Figure 2b, 2c). One strain (Figure 2d) displayed a thinly stained layer with ruthenium red with numerous "hairy" strands extending outward. The capsular antigen from this strain had been shown in a previous study (1) to be non-cross-reactive serologically when tested in agar gel diffusion with capsular antigens of two other strains of ss. asaccharolyticus against a rabbit antiserum to one of the other strains. In the current study, this strain has been shown to be strongly cross-reactive with the anti-capsular antiserum by use of the IFA test. This discrepancy may be due to the greater sensitivity of the IFA test. Clearly, there exists morphologic variation of capsular polysaccharide as demonstrated by electron microscopy.

No capsular polysaccharide was demonstrable in either of two strains of Bacteroides melaninogenicus ss. intermedius by electron microscopy with ruthenium red staining (Figure 2e, 2f); nor was capsular polysaccharide found in a strain of Bacteroides melaninogenicus ss. melaninogenicus (Figure 2g).

C. Summary

An indirect fluorescent antibody (IFA) test was developed using hyperimmune rabbit serum to a purified capsular polysaccharide of Bacteroides melaninogenicus subspecies asaccharolyticus. Twenty-three of twenty-three strains of B. melaninogenicus ss. asaccharolyticus were fluorescent-positive using this test. All eleven strains of B. melaninogenicus ss. intermedius tested and three strains of B. melaninogenicus ss melaninogenicus were fluorescent-negative. The IFA test demonstrated the presence of a subspecies specific capsular antigen from B. melaninogenicus ss. asaccharolyticus. The capsule was further identified by electron microscopy using ruthenium red, a polysaccharide-staining material.

D. Measurement of antibody to the capsular antigen by a quantitative enzyme-linked immunoabsorbent assay (ELISA).

a. Materials and Methods

Bacterial strains

Strains of Bacteroides melaninogenicus were obtained from sources

listed previously.

Preparation of antigen

Capsular polysaccharide was purified from a strain (381) of Bacteroides melaninogenicus subspecies asaccharolyticus according to methods detailed previously (1). For use in the ELISA, antigen was suspended in water and kept at 4° C until use.

Preparation of antiserum

New Zealand white rabbits were immunized with bacterial strains as described previously (1). Serum was stored frozen at -70° C until use.

Enzyme conjugated anti-immunoglobulin

Alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was conjugated to goat antiserum to rabbit IgG (H-chain specific) as outlined by Voller, et al. (6).

Enzyme-linked immunoabsorbent assay (ELISA)

Disposable polystyrene tubes 11 x 55 mm (Falcon, Berton, Dickinson & Co., Cockeysville, MD) were incubated with capsular antigen in a standard concentration in 1 ml of 0.05M carbonate buffer pH 9.6, containing 0.02% $NaN_3$ for 3hr at 37° C. The tubes were rinsed thoroughly with 0.15M NaCl containing 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO). Antiserum was diluted in phosphate-buffered saline (PBS), pH 7.3 and added in 1 ml quantities to each tube and incubated for 5 hr at room temperature. The tubes were again rinsed and the enzyme conjugate was diluted 1:500 in PBS, added to each tube, and incubated overnight at room temperature. The tubes were again rinsed and substrate (p-nitrophenylphosphate), diluted in 1 ml of carbonate buffer was added to the tubes in 1 mg quantities along with 1 mM $MgCl_2$. The enzyme reaction was stopped after 60 min with 0.2 ml 1M NaOH. Optical density (O.D.) was determined on a Beckman spectrophotometer (Beckman Instruments, Fullerton, CA) at 400 nm with a tungsten light source. Control tubes included antigen plus enzyme-conjugate (no antibody), antibody plus enzyme (no antigen), and enzyme (no antigen or antibody).

Purification of IgG from rabbit antiserum and quantitation by quantitative precipitin analysis

Purified IgG was obtained from rabbit antiserum to four strains of B. melaninogenicus subspecies asaccharolyticus according to the method of Joustra and Lundgren (7). B-lipoprotein was extracted from the serum by centrifugation at 12,000 g for 30 min. Eight ml of serum was then applied to a QAE Sephadex A-50 ion exchange column (Pharmacia, Uppsala, Sweden) equilibriated with a buffer containing 2.88 g. ethylene diamine in 73 ml 1M acetic acid made up to 1L with distilled water, pH 7, ionic strength 0.1. The void volume was collected and concentrated on a PM-30 membrane (Amicon

Corp., Lexington, MA) to 5 ml. To determine purity, immunoelectrophoresis was done as follows. Glass slides were pre-coated with 0.5% agarose gel. Slides were then coated with 1% agarose suspended in buffer containing 20.62 g. sodium barbital, 3.69 barbituric acid and 1200 ml distilled water. The purified IgG pool was placed in the wall and electrophoresis was carried out at 40 mA/slide for 2 hrs. The troughs were then filled with goat anti-human gamma globulin, goat anti-human-IgM and goat anti-human IgG and allowed to incubate in a moist chamber overnight at room temperature. The slides were stained with Buffalo black dye.

The amount of antibody in the IgG pool was determined by means of quantitative precipitin analysis as described by Gotschlich et al. (8), using a purified capsular polysaccharide antigen from a strain of B. melaninogenicus subspecies asaccharolyticus.

### Statistical methods

The Mann-Whitney U-test for non-parametrically distributed data was utilized (9).

## Results

### Optimal antigen concentration

A range of antigen concentrations was tested with three dilutions of a rabbit antiserum against a strain of Bacteroides melaninogenicus subspecies asaccharolyticus, in order to determine the optimal antigen concentration to be used in the ELISA. Figure 3 shows the pattern of O.D.'s derived from these studies.

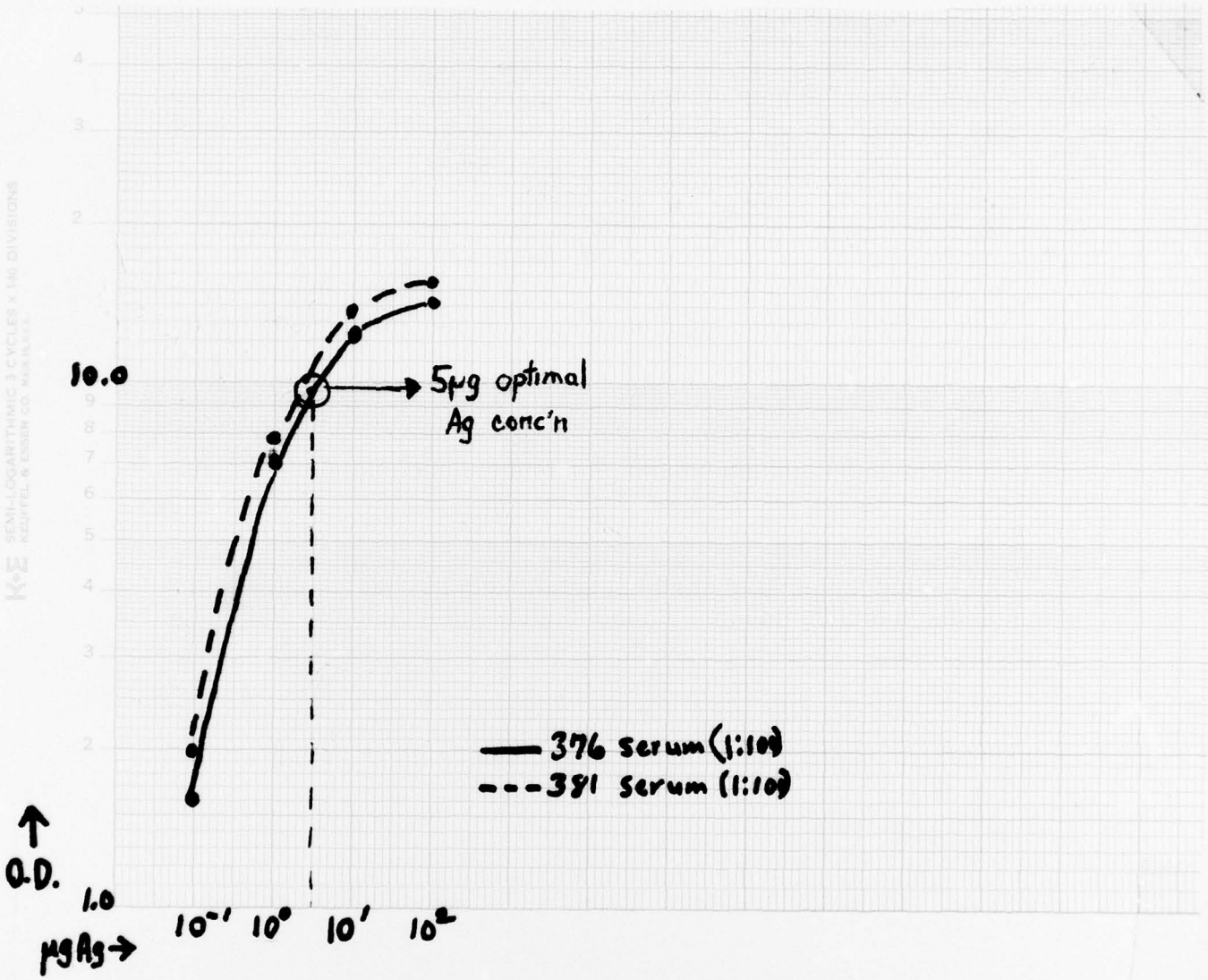


Figure 3. Curve relating antigen concentration (x-axis) to optical density (y-axis) with two antisera of B. melaninogenicus subspecies asaccharolyticus.

A concentration of 5 μg/ml antigen was determined to be optimal inasmuch as it gave a high O.D. reading that fell within the linear range on the curve (Figure 3) relating to O.D. to antigen concentration, i.e. a reproducible reading.

Titration of Antiserum

To be able to measure the quantity of antibodies in a particular serum in comparison with any number of other sera, a specific standard dilution was chosen. Figure 4 shows the range of O.D.'s for various dilutions of rabbit antiserum to three different strains of B. melaninogenicus subspecies asaccharolyticus when tested with a standard antigen concentration of 5 µg/ml.

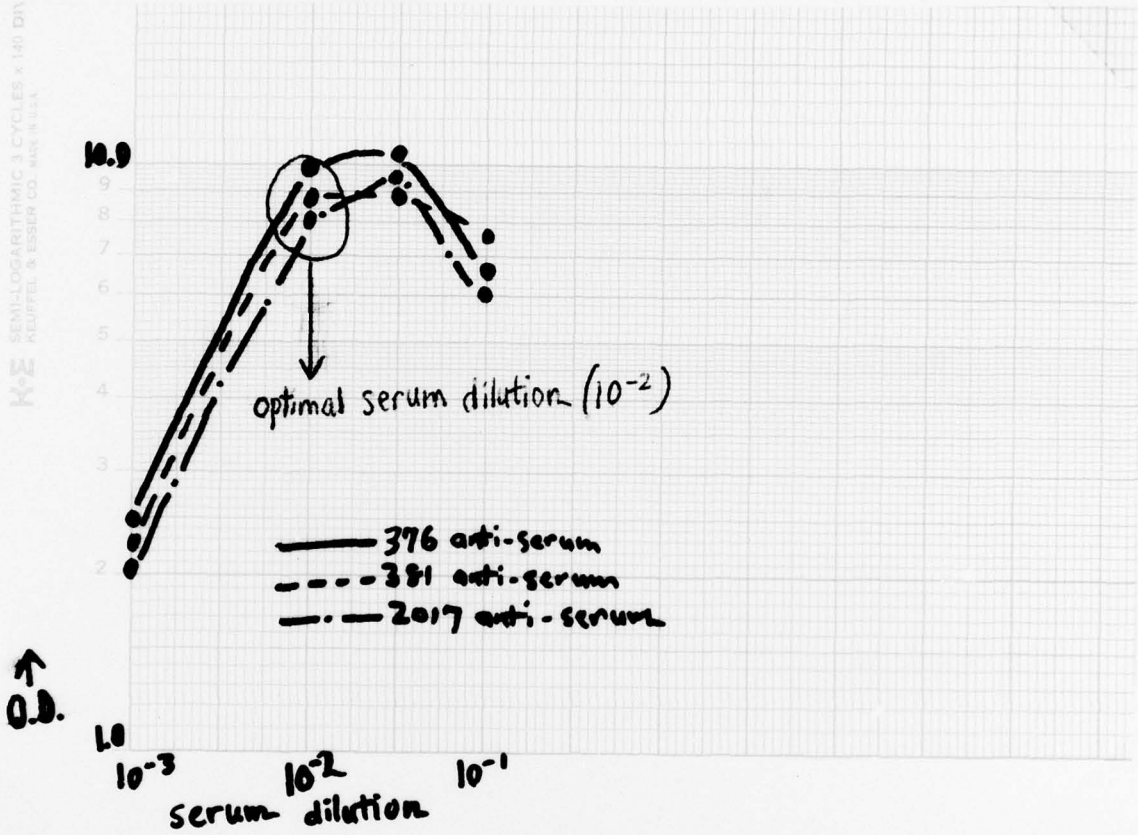


Figure 4. ELISA done with rabbit antisera from three strains of B. melanino-genicus subspecies asaccharolyticus using a standard antigen concentration of 5 µg/ml.

As the serum dilution decreases (i.e. antibody quantity increases), the curve relating antibody concentration to O.D. levels off. This phenomenon is due partly to a zone of antibody excess. A dilution of $10^{-2}$ (1:100) was chosen for comparison of antibody titers in various sera because at $10^{-2}$ dilution there exists a direct (linear) relationship between O.D. and quantity of antibody.

Measurement of anticapsular IgG in rabbit sera

Rabbit antiserum to fifteen strains of B. melaninogenicus subspecies asaccharolyticus, three strains of B. melaninogenicus subspecies intermedius and melaninogenicus, and several strains of other bacterial species were tested by the ELISA. Specific IgG in each serum directed against a capsular antigen from a strain of B. melaninogenicus subspecies asaccharolyticus was compared. The results are tabulated in Table 2.

Table 2. ELISA results of rabbit antisera to 15 strains of B. melaninogenicus subspecies asaccharolyticus, strains of B. melaninogenicus subspecies intermedius, strains of B. melaninogenicus subspecies melaninogenicus and several strains of other bacterial species. All sera were diluted 1:100. Antigen concentration was 5 µg/ml. Numbers refer to optical density (O.D.) after 1 hour incubation.

| | STRAIN | O.D. |
|---|---|---|
| 1. | Bacteroides melaninogenicus ss. asaccharolyticus | 7.45 |
| 2. | Bacteroides melaninogenicus ss. asaccharolyticus | 5.61 |
| 3. | Bacteorides melaninogenicus ss. asaccharolyticus | 7.78 |
| 4. | Bacteroides melaninogenicus ss. asaccharolyticus | 7.56 |
| 5. | Bacteroides melaninogenicus ss. asaccharolyticus | 6.98 |
| 6. | Bacteroides melaninogenicus ss. asaccharolyticus | 5.84 |
| 7. | Bacteroides melaninogenicus ss. asaccharolyticus | 7.64 |
| 8. | Bacteroides melaninogenicus ss. asaccharolyticus | 8.63 |
| 9. | Bacteroides melaninogenicus ss. asaccharolyticus | 7.82 |
| 10. | Bacteroides melaninogenicus ss. asaccharolyticus | 4.23 |
| 11. | Bacteroides melaninogenicus ss. asaccharolyticus | 8.46 |
| 12. | Bacteroides melaninogenicus ss. asaccharolyticus | 8.93 |
| 13. | Bacteroides melaninogenicus ss. asaccharolyticus | 8.91 |
| 14. | Bacteroides melaninogenicus ss. asaccharolyticus | 7.00 |
| 15. | Bacteroides melaninogenicus ss. asaccharolyticus | 6.93 |
| 16. | Bacteroides melaninogenicus ss intermedius | .25 |
| 17. | Bacteroides melaninogenicus ss. intermedius | .60 |
| 18. | Bacteroides melaninogenicus ss. intermedius | .72 |
| 19. | Bacteroides melaninogenicus ss. melaninogenicus | .40 |
| 20. | Bacteroides fragilis | .47 |
| 21. | Bacteroides fragilis | .47 |
| 22. | Bacteroides fragilis | .34 |
| 23. | Bacteroides fragilis | .28 |
| 24. | Bacteroides distasonis | .13 |
| 25. | Bacteroides vulgatus | .38 |
| 26. | Bacteroides Thetaiotaomicron | .05 |
| 27. | Bacteroides Thetaiotaomicron | .04 |
| 28. | Group B Streptococcus | .50 |
| 29. | Nigonorrheae | .45 |

A significantly higher amount of anti-capsular IgG was detectable in antisera to strains of B. melaninogenicus subspecies asaccharolyticus using a capsular antigen from a strain of this subspecies in the ELISA. Very low levels of specific anticapsular IgG were found in all of the sera not belonging to B. melaninogenicus subspecies asaccharolyticus. This finding corroborates a similar observation showing the presence of a cross-reactive capsular antigen that is specific to B. melaninogenicus subspecies asaccharolyticus in the indirect fluorescent antibody test.

E. Summary

The enzyme-linked immunoabsorbent assay has been developed to measure specific IgG directed against a subspecies specific capsular antigen of B. melaninogenicus subspecies asaccharolyticus. With this test we have shown that hyperimmune rabbit serum to strains of the subspecies all contain significant measurable IgG directed against a capsular antigen from one strain. Almost no specific IgG was detected in antiserum made against strains of the other two subspecies (intermedius and melaninogenicus), or in antisera made against several other bacterial species.

F. Immunization of gnotobiotic rats with purified outer membrane of Bacteroides melaninogenicus subspecies asaccharolyticus and measurement of antibody by the ELISA.

A. Materials and Methods

Gnotobiotic rat studies

Germ-free rats of the Sprague-Dawley strain were maintained in isolators and fed Keyes diet 2000 (10) fortified with vitamins (11) and sterilized with gamma radiation. At one month of age a serum sample was obtained from each rat by intracardiac puncture after sedation with intraperitoneal sodium pentobarbital. One group of six rats was then immunized with purified outer membrane (OM) from a strain of Bacteroides melaninogenicus subspecies asaccharolyticus as follows. Fifty µg OM, suspended in 0.1 ml PBS, pH 7.0, was given intramuscularly (i.m.) three times per week for two weeks. A booster dose was given in the fourth week. Fifty µg OM was mixed with 50 µg methylated bovine serum albumin in 0.1 ml PBS and given along with 0.1 ml Freund's complete adjuvant by i.m. injection in the first, second, and fourth weeks. The rats were bled in the fifth week. A group of 6 control rats were kept in a separate isolator and treated identically to the experimental group, except that 0.9% NaCl was substituted for OM as the immunogen. Sera were stored at -70° C until use. Survey cultures were taken at regular intervals to assure complete sterility within the isolators.

Antibody measurement by ELISA

Determination of anti-capsular IgG was performed by the ELISA technique, as described before, using alkaline phosphatase conjugated to anti-rat IgG goat globulin.

## Results

### Immunization studies in gnotobiotic rats

An outer membrane preparation from Bacteroides melaninogenicus subspecies asaccharolyticus was tested for immunogenicity in a group of germ-free rats. One group of six animals was immunized with purified outer membrane; a control group of the same size was immunized with 0.9% NaCl. Before and after immunization, antibody to the capsular polysaccharide of a heterologous (cross-reactive) strain of B. melaninogenicus subspecies asaccharolyticus was measured by the ELISA method. The results are tabulated in Table 3.

Table 3. Levels of anti-capsular IgG from rats immunized with purified outer membrane from Bacteroides melaninogenicus subspecies asaccharolyticus (Group A) compared to controls immunized with saline (Group B).

| | Group A | | | | Group B | | |
|---|---|---|---|---|---|---|---|
| Rat # | pre-immunization | post-immunization | post-minus pre- | Rat # | pre-immunization | post-immunization | post-minus pre- |
| 1 | 0.34* | 1.9 | 1.56 | 1 | .59 | .002 | -0.58 |
| 2 | 0.25 | 2.0 | 1.75 | 2 | .07 | .53 | 0.46 |
| 3 | 0.27 | 2.5 | 2.23 | 3 | .10 | .24 | 0.14 |
| 4 | 0.4 | 1.0 | 0.6 | 4 | .041 | .18 | 0.14 |
| 5 | 0.53 | 1.8 | 1.27 | 5 | .045 | .125 | 0.08 |
| 6 | 0.27 | 2.1 | 1.83 | 6 | .057 | .014 | -0.043 |

* Number refers to O.D. measured at 400 nm; all sera were tested at 1:500 dilution.

All six rats immunized with outer membrane showed a significant rise in antibody titer in comparison to preimmunization levels in comparison to the saline-immunization group ($p<0.001$). The absolute amount of specific anticapsular IgG was not determined. Comparisons in antibody titer between experimental and control groups were accomplished by measuring the O.D. at a certain time (60 min) for a specific serum dilution.

## Summary

We have shown in this preliminary set of experiments that it is possible to induce an antibody response in a germ free rat model by immunizing with purified outer membrane material from a strain of B. melaninogenicus subspecies asaccharolyticus. Furthermore, antibody is measurable using very small amounts of serum by a sensitive, specific ELISA technique.

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